A method to establish co-cultures of myotubes and preadipocytes from collagenase digested neonatal pig semitendinosus muscles[1]

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ABSTRACT: The relationships between adipocyte and muscle cell development within muscle are important in the study of factors or agents that may improve meat quality. Neonatal porcine muscle has the potential to yield both cell types for cell culture because it contains developing adipocytes and a high number of muscle satellite cells. Therefore, we modified a conventional collagenase-based procedure to digest neonatal porcine muscle and subsequently cultured the resultant muscle stromal-vascular (SV) cells on several substrata in basal and dexamethasone (DEX)-containing media. Developing myotubes and preadipocytes were present in muscle SV cell cultures on laminin substrata following seeding and plating with fetal bovine serum (FBS) with or without DEX. Myotube number was much higher ($P < 0.05$) on laminin substrata compared with all other substrata, whereas preadipocyte number in muscle SV cell cultures was independent of substrata, as we have shown previously. This approach can be used to establish co-cultures of differentiating adipocytes and myotubes from collagenase-digested neonatal pig muscle. Because the comparison is within the same culture dish, this method allows for a direct comparison of the responses of adipogenic and myogenic cells to growth and differentiation factors. For example, DEX did not alter myogenesis (i.e., 11 ± 3 vs. 11 ± 4 myotubes per unit area for control and DEX-treated cultures, respectively), but it has been shown to markedly increase preadipocyte number in muscle SV cell cultures.

Key Words: Cell Biology, Cell Culture, Laminin, Myotube, Preadipocyte

Introduction

Cell cultures of established cell lines are widely used but they are limited because cells are usually cultured in complex environments, and the results may not represent what occurs in vivo. In contrast, primary cell cultures are more likely to represent the in vivo condition because cells are removed directly from the animal and grown in relatively simple culture environments. However, primary cell cultures have not been able to mimic certain cell-to-cell interactions or paracrine/autocrine relationships that can be examined in co-cultures.

Protocols of both cell line and primary cell cultures are usually designed for optimal yield and subsequent growth and differentiation of a particular cell type. For instance, collagenase digestion is conventionally used to liberate preadipocytes and other cells from adipose tissue (adipose tissue stromal-vascular [**SV**] cells; Hausman, 1989), whereas trypsin or pronase are typically used to liberate myoblasts or muscle satellite cells from muscle (Hembree et al., 1991; Dodson et al., 1996). Conceivably, collagenase digestion of muscle could liberate both preadipocytes (SV cells) and myogenic or satellite cells, thereby allowing co-culture of these cell types. This paper describes simple procedures to effectively create co-cultures of primary myotubes and i.m. (muscle) preadipocytes following collagenase digestion of neonatal porcine muscle.

Materials and Methods

Isolation of Primary Stromal-Vascular Cells from Semitendinosus Muscles

All solutions were warmed to 37°C before use. Basal medium (Dulbecco's modified Eagle's medium; **DMEM**) consisted of DME/F12 medium (Sigma-Aldrich Co., St. Louis, MO) supplemented with 2.5 µg/L amphotericin B (Sigma-Aldrich Co.), 50 µg/L Cephazolin (American Pharmaceutical Partners, Inc., Schaumburg, IL), and

50 μg/L Gentamicin (Vedco, Inc., St. Joseph, MO). Five- to seven-day-old pigs were killed via an i.p. injection of 3 g of sodium pentobarbitol (10% wt/vol solution; Butler Co. Columbus, OH) followed by exanguination. A total of 13 muscle SV cell cultures, representing 13 pigs, were established and examined in these studies. Aseptic techniques in a biological safety cabinet were used for remaining procedures. Skin and connective tissue were removed from both hind limbs to expose semitendinosus muscles. Both semitendinosus muscles, weighing approximately 8 g, were isolated and removed. Tissue was finely minced in 50-mL beakers containing 5 mL of DMEM with 0.2 mL of kanamycin solution (Sigma-Aldrich Co.) per gram of tissue. Tissues were transferred to eight digestion flasks (Fisher Scientific, Hampton, NH) and subjected to a 2-h digestion at 37°C in a shaking water bath without regulated O_2/CO_2 atmosphere. The digestion buffer included 100 mM HEPES (Sigma Aldrich Co.) buffer containing 120 mM NaCl, 50 mM KCl, 5 mM D-glucose, 1.5% (wt/vol) type-V BSA (Sigma-Aldrich Co.), 1 mM $CaCl_2$, and a type-II collagenase (Sigma-Aldrich Co.) solution. The type-II collagenase solution used to isolate stromal-vascular cells from semitendinosus muscle contained approximately 12,500 collagen digestion units per tube. The semitendinosus muscle digest was centrifuged at $500 \times g$ for 5 min and filtered through 180- and 20-μm nylon mesh filters (Millipore Corp., Billerica, MA) into 50-mL centrifuge tubes. Cells were rinsed with 30 mL of DMEM and centrifuged at $1,500 \times g$ for 10 min. Media were aspirated, cell pellets combined, and cells were rinsed with another 30 mL of DMEM. This was followed by centrifugation at $1,500 \times g$ for 10 min, and media were aspirated. The cell pellet was resuspended in 10 mL of DMEM, and viable cells were quantified using a hemocytometer (Fisher Scientific) and 0.4% trypan blue stain (Invitrogen Corp., Carlsbad, CA). Stromal-vascular cells (5×10^5) were seeded and plated in either noncoated dishes (BD Biosciences, Bedford, MA), or dishes precoated with laminin, type IV collagen, or fibronectin (BD Biosciences) in 2 mL of DMEM with10% fetal bovine serum (**FBS**) ± 80 nM dexamethasone (**DEX**) media per dish. Cells were incubated in a 5% CO_2, 100% humid incubator at 37°C (NuAire Inc., Plymouth, MN). Media were aspirated 1 h after plating, and 2 mL of fresh media were added to each dish. Preadipocytes attach within 1 h of plating (Yu and Hausman, 1998), whereas myogenic cells attach much more slowly (Hembree et al., 1991), so dishes were rinsed 1 h after plating to increase the relative number of preadipocytes and decrease the number of myogenic cells. The "unattached" cells were reseeded into laminin-coated and uncoated dishes following the 1-h rinse.

Experimental Treatments and Design

Cells in media containing 10% FBS ± DEX reached confluence 3 to 5 d after plating. At confluence, cell cultures were rinsed for 10 min in DMEM and shifted to serum-free media containing 5 U/mL of bovine insulin, 5 μg/mL of human transferrin, and 5 ng/mL of selenium (**ITS**; Sigma-Aldrich Co.) for 2 to 3 d. Therefore, two treatments were used in these experiments: 1) FBS + DEX on d 0 to 3, followed by ITS on d 3 to 6; and 2) FBS on d 0 to 3, followed by ITS on d 3 to 6, which represented the control or no DEX treatment.

Myotubes were counted in two studies of muscle SV cells cultured on laminin, type IV collagen, fibronectin, or no substrata and treated with DEX. Cultures were rinsed after the first hour and treated with serum-free media with ITS from d 3 to 6 following FBS ± DEX from d 0 to 3. Regardless of substrata, DEX treatment had no influence on myotube number and, compared with laminin, there were negligible numbers of myotubes in DEX-treated type IV collagen, fibronectin, and uncoated cultures (i.e., myotubes per unit area were 1.1 ± 0.5 for fibronectin, 0.3 ± 0.2 for type IV collagen, and 0.3 ± 0.1 for uncoated dishes; means ± SEM of two studies). Therefore, no other DEX studies were performed with type IV collagen and fibronectin substrata, and only laminin and uncoated substrata were used in subsequent studies.

Cytochemistry for Lipid Content Determination

Culture media were aspirated from culture dishes, and cells were rinsed with PBS followed by fixation in 4% paraformaldehyde (Sigma-Aldrich Co.) for 30 min at room temperature. Paraformaldehyde was removed and dishes rinsed twice with PBS. Culture dishes were then incubated in 60% Oil-Red-O solution (Sigma-Aldrich Co.) for 10 min followed by a 3-min rinse in distilled, deionized water. Cover slips were mounted with glycerol gelatin (Sigma-Aldrich Co.).

Immunocytochemistry

Six days after plating, cell cultures were incubated with primary antibodies for peroxisome proliferator activated receptor-γ (**PPARγ**), CCAAT/enhancing binding protein-α (**C/EBPα**), desmin, vimentin, and a monoclonal antibody designated AD-3 (Yu and Hausman, 1998) followed by visualization of reactivity with extravidin peroxidase staining kits (Sigma-Aldrich Co.). The AD-3 monoclonal antibody and C/EBPα and PPARγ antibodies were used to stain for preadipocytes and adipocytes, whereas desmin and vimentin were used to identify myoblasts and myotubes. As described by Hausman and Poulos (2004), cultures also were stained with the 5.1H11 monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City), which recognizes a cell surface antigen on myoblasts and myotubes. Some cultures were incubated with AD-3 antibody on d 3 to 5 before shifting to serum-free media. Briefly, cells were fixed for 30 min using 4% paraformaldehyde, permeabilized with 3% (vol/vol) Triton X-100 (Sigma-Aldrich Co.) for 15 min for PPARγ, C/EBPα, desmin, and vimentin and incubated

with antibodies for 1 h at room temperature in a humidity chamber. Desmin (sc7556), PPARγ (sc7273), C/EBPα, (sc61), and vimentin (sc6260) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and were diluted to 1/200, 1/50, 1/50, and 1/50, respectively. In six experiments, counterstaining was not done following PPARγ and C/EBPα immunocytochemistry to provide optimal contrast to enhance discrimination between cell types. Double staining was done in seven experiments, and it simply involved Oil-Red-O staining (no additional fixation) after peroxidase staining for PPARγ, C/EBPα, AD-3, or the 5.1H11 antigen. Oil-Red-O staining was used to visualize cellular lipid accumulation, and cultures were counterstained with hematoxylin (Anatech, LTD, Battle Creek, MI) to visualize cell number and morphology. Stained cells were visualized using a microscope, IX51 with digital photomicrographic system (Olympus America, Melville, NY).

## Evaluation of Fat Cell Cluster, Myotube, and Total Cell Number

Cultures were routinely stained for lipid and counterstained as detailed elsewhere (Hausman, 1981). Three photomicrographs of each vessel were used for total cell counting. Fat cell clusters (≥ 3 fat cells) and myotubes (≥ 3 nuclei) were counted in 6 mm^2 and 3.5 mm^2 microscopic fields, respectively, and six to eight microscopic fields of each dish were counted.

Statistical Analyses

Data were subjected to a one-way ANOVA procedure of SAS (SAS Inst., Inc., Cary, NC) to determine the main effect of substrata. Differences between means were determined by least squares contrast statements of the ANOVA procedure. Comparison of two means was accomplished by a Student's t-test (Steel and Torrie, 1960).

Results and Discussion

We initiated studies to culture preadipocytes from porcine muscle (i.e., i.m. preadipocytes) by digesting muscles cleaned of all connective tissue with a collagenase-based procedure used to isolate and culture adipose tissue SV cells. Primary cultures of porcine adipose tissue SV cells primarily contain preadipocytes and fibroblasts. Because we anticipated a relatively low number of preadipocytes in i.m. (muscle) SV cell cultures, we used dishes precoated with extracellular matrix substrata including laminin because it preferentially enhances preadipocyte attachment and spreading (Hausman et al., 1996). We anticipated little to no myotube formation in these cultures because a low yield of myogenic cells was expected, and our postconfluent serum-free media (ITS) was optimal for preadipocyte lipid accretion (Hausman, 1989). Regardless, myotubes consis-
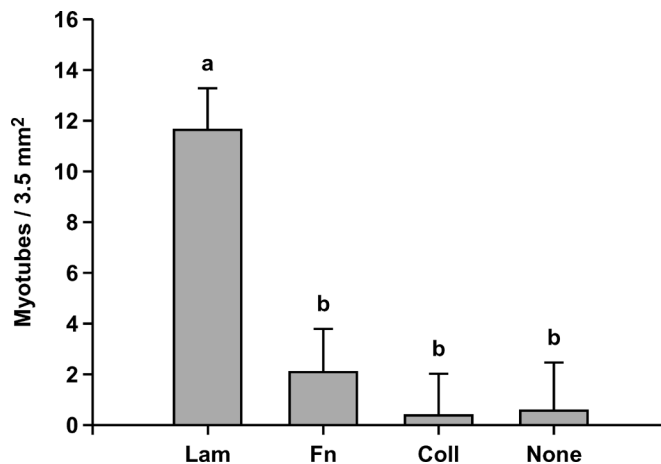

Figure 1. Myotube number in muscle stromal-vascular cell cultures on various substrata. Cultures were rinsed after the first hour, treated with serum-free media with insulin, transferrin, and selenium (ITS) from d 3 to 6 (following fetal bovine serum from d 0 to 3), and stained with hematoxylin on d 6. Values are least squares means $\pm$ SEM for three to six experiments with three to four dishes in each experiment. Letters above bars (a, b) indicate that laminin (Lam) substrata increased myotube number per unit area relative to fibronectin (Fn), type IV collagen (Coll), and no (None) substrata, $P < 0.05$.

tently developed in confluent muscle SV cultures on laminin substrata after switching to ITS. Myotubes did not develop in subconfluent cultures or in cultures continuously treated with serum (FBS d 0 to 6) or no serum (ITS d 0 to 6). Myotube formation in muscle SV cell cultures was greater ($P < 0.05$) on laminin substrata than on other substrata (Figure 1), whereas fat cell cluster development in control media (no DEX) was independent of substrata (i.e., fat cell clusters per unit area were 1.9 ± 0.4 for laminin-coated dishes and 1.4 ± 0.3 for all other substrata; means $\pm$ SEM of seven to 14 studies).

Treatment with DEX had no influence on myogenesis in laminin-coated dishes (i.e., myotubes per unit area were 11 ± 3 in control [no DEX] cultures and 11 ± 4 in FBS+DEX-treated cultures; means $\pm$ SEM of four to six studies). In contrast, FBS+DEX increased fat cell cluster numbers fourfold in muscle SV cultures in laminin coated dishes (Hausman and Poulos, 2004).

Myotubes in muscle SV cultures on laminin substrata were immunoreactive for desmin (not shown) and vimentin (Figure 2). Furthermore, immunostaining with the 5.1H11 antibody clearly distinguished myoblasts and small and larger myotubes from preadipocytes and other cells (Figure 3). Collective observations of cultures either counterstained or not counterstained clearly showed that myoblasts and myotubes were not reactive for the AD-3 antigen, C/EBPα, or PPARγ and contained no lipid (Figure 4). Furthermore, preadipo-
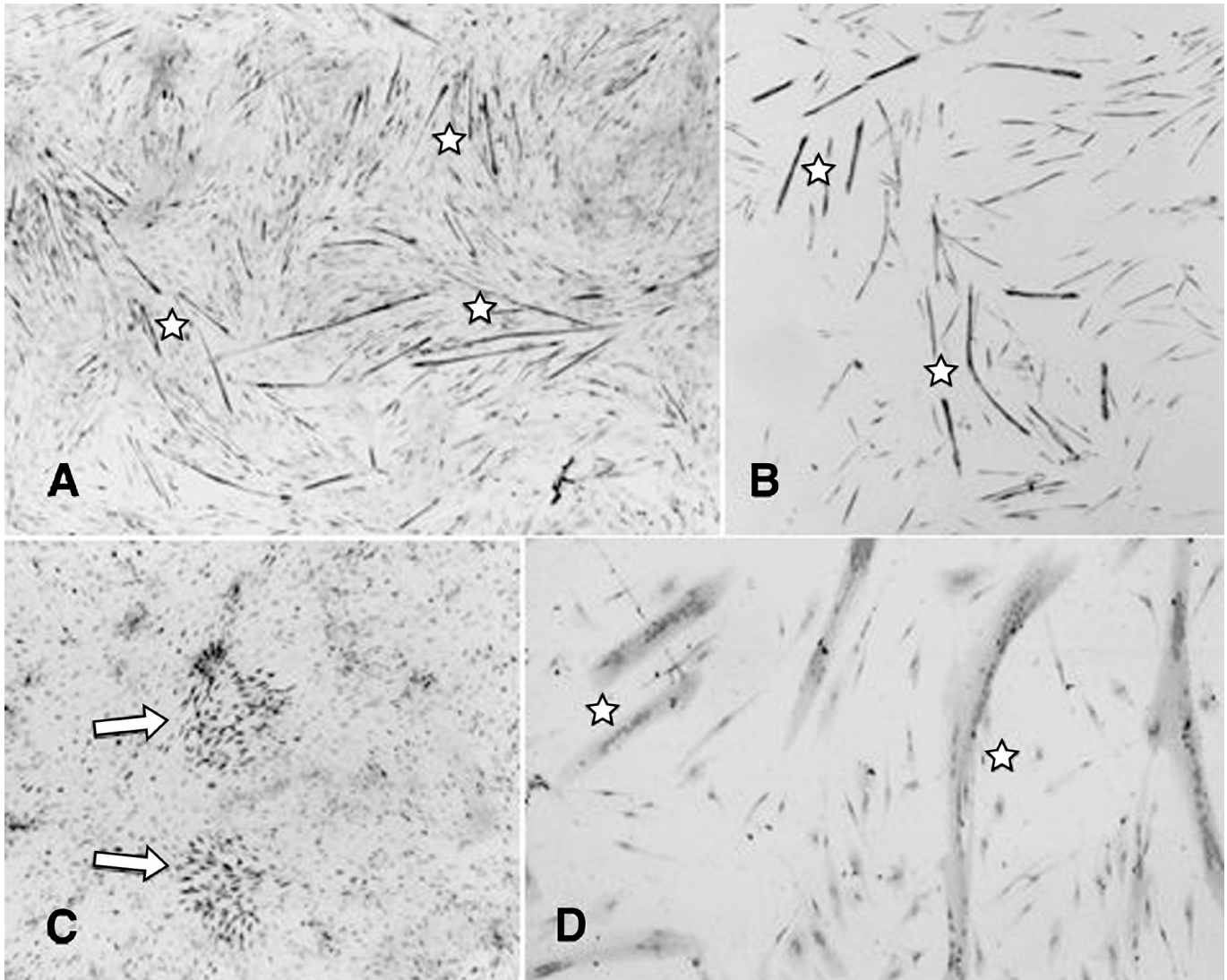
Figure 2. Survey micrographs of typical muscle stromal-vascular cells from the same digest cultured on either laminin (A and B) or no substrata (C). One digest was used to establish three types of cultures: a culture on laminin that was rinsed after the first hour (A); an unattached cell culture that contained cells that did not attach by the first hour which were then reseeded on laminin (B); and a culture on uncoated substrata that was rinsed after the first hour (C). From another digest, stromal-vascular cells that did not attach after 1 h were reseeded on laminin (D, unattached cell culture). Cultures were either stained for vimentin (A, B, and C) or hematoxylin and oil red O (D) after serum-free treatment from d 3 to 6 with insulin, transferrin, and selenium (ITS) following fetal bovine serum from d 0 to 3. Immunoreactivity was visualized by using a peroxidase staining kit. Cultures on laminin from the same digest (A and B) had similar numbers of myotubes (stars), but the proportion of myotubes relative to total cell number was much greater in the unattached cell culture (B). Note the large multinucleated myotubes (stars) in the culture of unattached cells (D). Clusters of vimentin-reactive cells with no myotubes were evident in cultures on uncoated substrata (C, arrowheads). For A, B, and C, magnification = 65×; for D, magnification = 140×.

cytes with or without lipid were stained with the AD-3 antibody, whereas only preadipocytes with lipid had PPARγ immunoreactive nuclei (Figure 4).

The relative number of myotubes in muscle SV cultures was increased when cells unattached after 1 h were reseeded in laminin-coated dishes, regardless of media treatment (Figure 2). The percentage of total nuclei that were myotube nuclei was 49 ± 1 for cultures of cells that were unattached (after 1 h) and reseeded

in laminin dishes compared with 11.4 ± 3 for rinsed muscle cultures on laminin ($P < 0.05$; means ± SEM; n = 2 to 6 experiments). In contrast, the percentage of total nuclei that were myotube nuclei was less than 1% in rinsed muscle SV cultures on uncoated or fibronectin substrata (Hausman and Poulos, 2004). Furthermore, reseeding unattached cells in uncoated or fibronectin-coated dishes did not increase the relative number of myotubes in muscle SV cultures.
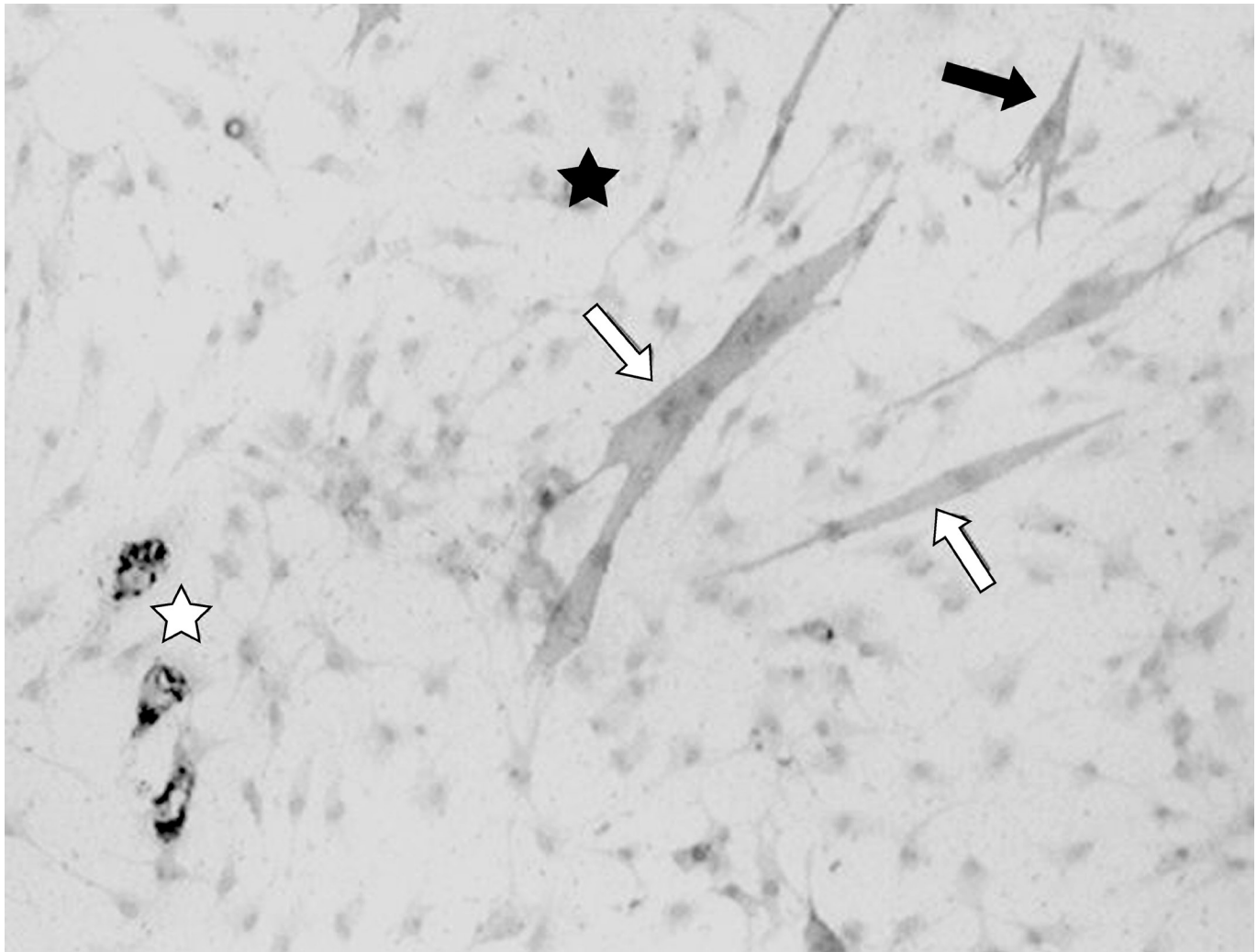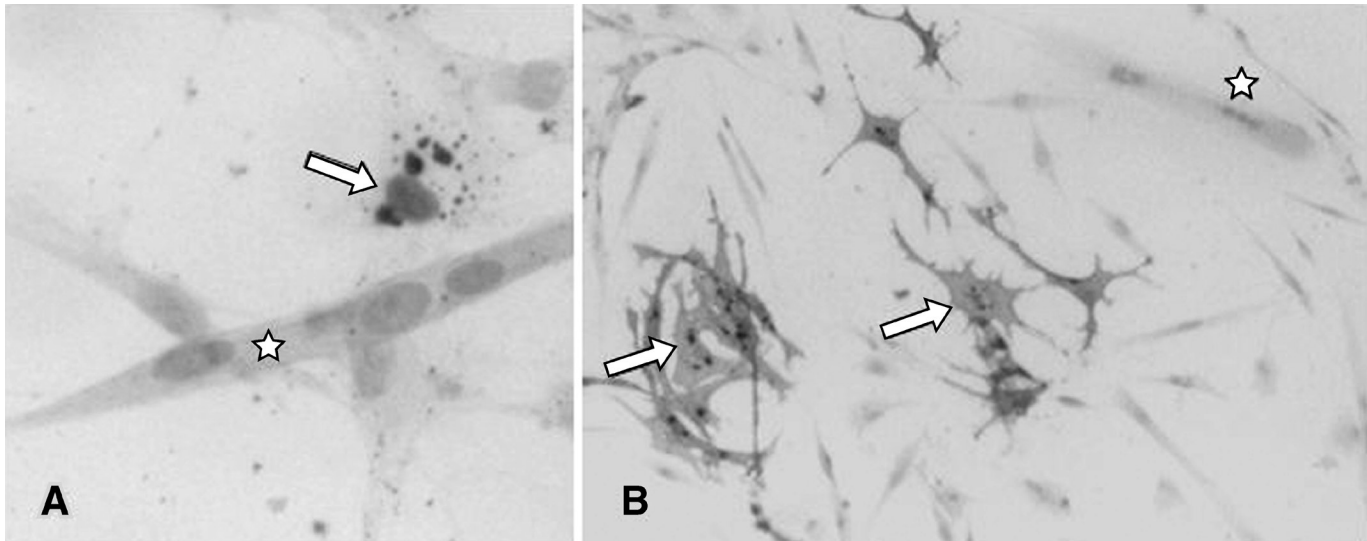
Figure 3. A typical muscle culture of stromal-vascular cells cultured on laminin, rinsed after the first hour and treated with serum-free media with insulin, transferrin, and selenium (ITS) from d 3 to 6 following fetal bovine serum + dexamethasone from d 0 to 3. Immunostaining with the 5.1H11 monoclonal antibody, a myoblast/myotube marker, and counterstaining with oil red O and hematoxylin was done on d 6. Immunoreactivity was visualized by using a peroxidase staining kit. Myoblasts (black arrow) and small and larger myotubes (white arrows) are clearly delineated by 51H11 antigen reactivity, whereas preadipocytes (white star) and other stromal cells (black star) are not delineated. Lipid accretion was evident in preadipocytes and appears as the dark cytosolic material (white star). Magnification = 200×.

We did not adjust the number of cells that were unattached after 1 h and reseeded in laminin dishes. Unattached cells from one dish were simply transferred or reseeded in another laminin-coated dish. Thus, some dishes had lower total cell numbers than did others within an experiment. Myotube size and the absolute myotube number varied from dish to dish, reflecting variability in initial cell numbers; however, myonuclei per total nuclei varied much less, indicating that the rinsed cells were primarily myogenic, regardless of initial cell numbers. The substrata for the first hour had no influence on subsequent cultures of cells that were unattached and reseeded in a laminin dish. Thus, it is necessary that rinsed cells be reseeded in a laminin-coated dish if cultures enriched for myogenic cells are desired. Rinsing (after 1 h) did not influence the percentage or absolute number of preadipocytes.

Methods have been developed to co-culture either 3T3-L1 preadipocytes with muscle satellite cells (Dodson et al., 1997; Hossner et al., 1997) or human skeletal muscle cells with human adipocytes (Dietze et al., 2002). However, the present study is the first report of a method to establish primary cultures that contain differentiating adipocytes and myotubes representing the same animal and tissue. Our "co-cultures" mimic the close anatomical relationships between adipocytes and muscle fibers observed in developing pig muscle (Gondret and Lebret, 2002). The utility of these co-cultures is demonstrated in several ways. When coupled with immunocytochemical techniques, they can be used

Figure 4. Muscle stromal-vascular cells cultured on laminin, rinsed after the first hour and treated with serum-free media with insulin, transferrin, and selenium (ITS) from d 3 to 6 following either fetal bovine serum + dexamethasone (A) or pig serum (B) from d 0 to 3. On d 6, cultures were either stained with a peroxisome proliferator activated receptor γ (PPARγ) antibody (A) or the AD-3 monoclonal antibody (B), a preadipocyte marker, and counterstained with oil red O and hematoxylin. Immunoreactivity was visualized by using a peroxidase staining kit. Note that the nuclei in a small myotube (A, star) were not PPARγ immunoreactive, in contrast to PPARγ nuclear immunoreactivity (A, white arrow) in a preadipocyte with lipid. Immunoreactivity for the AD-3 antigen delineates preadipocytes with little to no lipid (B, white arrows). Note the absence of AD-3 reactivity in a small myotube (B, star). Panel A magnification = 640×; Panel B magnification = 125×.

to evaluate critical quantitative and qualitative aspects of the response of myogenic cells and muscle to PPARγ ligands highlighted in recent studies (Park et al., 1998; Kausch et al., 2001; Holst et al., 2003). For instance, do PPARγ ligands influence either the number of myotubes formed or the number of differentiated preadipocytes? Furthermore, do PPARγ ligands activate PPARγ in muscle cells and/or associated fibroblasts and preadipocytes? We demonstrated herein that PPARγ is not expressed by the nuclei of fibroblasts, myoblasts, or myotubes, but it is expressed by the nuclei of preadipocytes with lipid (Figure 4). Similar comparisons of these cell types can be made with regard to the expression of other transcription factor proteins and regulatory proteins.

Studies of semitendinosus muscle cells from 1-wk-old pigs (Mesires and Doumit, 2002) indicate that the majority of myogenic cells in the present study cultures are probably satellite cells. Procedures for culturing muscle satellite or myogenic cells from most species involve many steps. Furthermore, myotube formation is induced with either serum supplemented media or serum-free media supplemented with insulin and dexamethasone or triiodothyronine and several other compounds (Doumit and Merkel, 1992; Cassar-Malek et al., 1999; Yi et al., 2001). In the present study, the use of laminin substrata and a simple commercially available serum-free media resulted in a method to induce myotube formation that is simpler and involves fewer steps. Furthermore, combining this method with culturing

cells unattached after 1 h resulted in myotube fusion percentages comparable to values reported for pig satellite cell and fetal pig myogenic cell cultures (Hembree et al., 1991; Fligger et al., 1998).

Implications

Fat cells in muscle, or marbling fat cells, develop later than other fat cells, and recent studies indicate that it may be possible to preferentially increase marbling fat cell development. Co-cultures of marbling fat cell precursors and developing muscle cells were used to compare the development of muscle cells and marbling fat cell precursors under identical conditions. These co-cultures can be used to examine developmental relationships between muscle cells and marbling fat cell precursors. Furthermore, they can be used to screen for dietary supplements or growth promotants that differentially influence muscle cell and marbling fat cell development.

Literature Cited

Cassar-Malek, I., N. Langlois, B. Picard, and Y. Geay. 1999. Regulation of bovine satellite cell proliferation and differentiation by insulin and triiodothyronine. Domest. Anim. Endocrinol. 17:373–388.

Dietze, D., M. Koenen, K. Rohrig, H. Horikoshi, H. Hauner, and J. Eckel. 2002. Impairment of insulin signaling in human skeletal muscle cells by co-culture with human adipocytes. Diabetes 51:2369–2376.

Dodson, M. V., D. C. McFarland, A. L. Grant, M. E. Doumit, and S. G. Velleman. 1996. Extrinsic regulation of domestic animal-derived satellite cells. Domest. Anim. Endocrinol. 13:107–126.

Dodson, M. V., J. L. Vierck, K. L. Hossner, K. Byrne, and J. P. McNamara. 1997. The development and utility of a defined muscle and fat co-culture system. Tissue Cell 29:517–524.

Doumit, M. E., and R. A. Merkel. 1992. Conditions for isolation and culture of porcine myogenic satellite cells. Tissue Cell 24:253–262.

Fligger, J. M., P. V. Malven, M. E. Doumit, R. A. Merkel, and A. L. Grant. 1998. Increases in insulin-like growth factor binding protein-2 accompany decreases in proliferation and differentiation when porcine muscle satellite cells undergo multiple passages. J. Anim. Sci. 76:2086–2093.

Gondret, F., and B. Lebret. 2002. Feeding intensity and dietary protein level affect adipocyte cellularity and lipogenic capacity of muscle homogenates in growing pigs, without modification of the expression of sterol regulatory element binding protein. J. Anim. Sci. 80:3184–3193.

Hausman, G. J. 1981. Techniques for studying adipocytes. Stain Technol. 56:149–154.

Hausman, G. J. 1989. The influence of insulin, triiodothyronine (T3) and insulin-like growth factor-I (IGF-1) on the differentiation of preadipocytes in serum-free cultures of pig stromal-vascular cells. J. Anim. Sci. 67:3136–3143.

Hausman, G. J., and S. Poulos 2004. Recruitment and differentiation of intramuscular preadipocytes in stromal-vascular cell cultures derived from neonatal pig semitendinosus muscles. J. Anim. Sci. 82:429–437.

Hausman, G. J., J. T. Wright, and R. L. Richardson. 1996. The influence of extracellular matrix substrata on preadipocyte development in serum-free cultures of stromal-vascular cells. J. Anim. Sci. 74:2117–2128.

Hembree, J. R., M. R. Hathaway, and W. R. Dayton. 1991. Isolation and culture of fetal porcine myogenic cells and the effect of insulin, IGF-I, and sera on protein turnover in porcine myotube cultures. J. Anim. Sci. 69:3241–3250.

Holst, D., S. Luquet, K. Kristiansen, and P. A. Grimaldi. 2003. Roles of peroxisome proliferator-activated receptors delta and gamma in myoblast transdifferentiation. Exp. Cell Res. 288:168–176.

Hossner, K. L., R. Yemm, J. Vierck, and M. V. Dodson. 1997. Insulin-like growth factor (IGF)-I and -II and IGFBP secretion by ovine satellite cell strains grown alone or in coculture with 3T3-L1 preadipocytes. In Vitro Cell Dev. Biol. Anim. 33:791–795.

Kausch, C., J. Krutzfeldt, A. Witke, A. Rettig, O. Bachmann, K. Rett, S. Matthaei, F. Machicao, H. U. Haring, and M. Stumvoll. 2001. Effects of troglitazone on cellular differentiation, insulin signaling, and glucose metabolism in cultured human skeletal muscle cells. Biochem. Biophys. Res. Commun. 280:664–674.

Mesires, N. T., and M. E. Doumit. 2002. Satellite cell proliferation and differentiation during postnatal growth of porcine skeletal muscle. Am. J. Physiol. Cell Physiol. 282:C899–C906.

Park, K. S., T. P. Ciaraldi, K. Lindgren, L. Abrams-Carter, S. Mudaliar, S. E. Nikoulina, S. R. Tufari, J. H. Veerkamp, A. Vidal-Puig, and R. R. Henry. 1998. Troglitazone effects on gene expression in human skeletal muscle of type II diabetes involve up-regulation of peroxisome proliferator-activated receptor-gamma. J. Clin. Endocrinol. Metab. 83:2830–2835.

Steel, R. G. D., and J. H. Torrie. 1960. Principles and Procedures of Statistics. McGraw-Hill Book Co., New York.

Yi, Z., M. R. Hathaway, W. R. Dayton, and M. E. White. 2001. Effects of growth factors on insulin-like growth factor binding protein (IGFBP) secretion by primary porcine satellite cell cultures. J. Anim. Sci. 79:2820–2826.

Yu, Z. K., and G. J. Hausman. 1998. Preadipocyte screening by laminin in porcine stromal vascular cell cultures. Obes. Res. 6:299–306.